Direct Analysis of Tubulin Expression in Cancer Cell Lines by Electrospray Ionization Mass Spectrometry†

Pascal Verdier-Pinard,‡,+ Fang Wang,§,+ Berta Burd,‡ Ruth Hogue Angeletti,§ Susan Band Horwitz,‡ and George A. Orr*,‡

Department of Molecular Pharmacology and Laboratory for Macromolecular Analysis and Proteomics, Albert Einstein College of Medicine, Bronx, New York 10461

Received June 13, 2003; Revised Manuscript Received August 14, 2003

ABSTRACT: Differential expression of tubulin isotypes, mutations, and/or post-translational modifications in sensitive and Taxol-resistant cell lines suggests the existence of tubulin-based mechanisms of resistance. Since tubulin isotypes are defined by their C-terminal sequence, we previously described a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based analysis of tubulin diversity in human cell lines by analysis of their CNBr-released C-terminal peptides [Rao, S., Aberg, F., Nieves, E., Horwitz, S. B., and Orr, G. A. (2001) *Biochemistry 40*, 2096−103]. We now describe the liquid chromatography/electrospray ionization mass spectrometry analysis of native tubulins in Taxol-stabilized microtubules from parental and Taxol/epothilone-resistant human cancer cell lines. This method allows the direct determination of tubulin isotype composition, including post-translational modifications and mutations occurring throughout the entire protein. Four major isotypes, βI-, βIVb-, Kα1-, and α6-tubulin, were detected in two human carcinoma cell lines, A549 and HeLa. βIII-Tubulin represented a minor species, as did α4-tubulin which was detected for the first time in both cell lines. The three α-tubulins were almost totally tyrosinated, and post-translational modifications were limited to low levels of monoglutamylation of Kα1-, βI-, and βIII-tubulin. βII- and βIVa-tubulins were not detected in either parental or drug-resistant cell lines, in contrast to previous RNA-based studies. Since mutations can occur in a single tubulin allele, the question as to whether the wild-type and mutant transcripts are both translated, and to what levels, is important. Heterozygous expression of Kα1- or βI-tubulin mutants that introduced mass changes as small as 26 Da was readily detected in native tubulins isolated from Taxol- and epothilone-resistant cell lines.

Microtubules composed of α/β-tubulin heterodimers are involved in a diverse range of cellular functions including motility, morphogenesis, intracellular trafficking of macromolecules and organelles, and mitosis and meiosis (*1*−*3*). This functional diversity is achieved through the association of structural and motor MAPs[1] with microtubules. MAPs have the ability to regulate the dynamic organization and stability of microtubules and also to recruit other proteins to the microtubule cytoskeleton. The multiple α- and β-tubulin isotypes (each ∼450 amino acids), although highly conserved, display extensive sequence variations at their C-termini which participate in the binding of MAPs to microtubules (*4*). Additionally, tubulins are post-translationally modified by polyglutamylation, polyglycylation, and phosphorylation on both α- and β-subunits, and by acetylation, detyrosination/tyrosination, and removal of the penultimate glutamic acid residue on α-tubulins (*4*, *5*). The functional significance of this structural diversity remains unclear, except that specific isotypes are incorporated into microtubule-based organelles, such as centrioles, and that differential expression of tubulin isotypes has been observed between tissues and during development (*4*). The highly divergent C-termini may provide a mechanism for isotype-specific MAP binding. Although MAPs generally regulate their binding affinity for microtubules by phosphorylation, there is an increasing body of evidence suggesting that post-translational modifications of α/β-tubulin can also regulate the association of MAPs with microtubules (*6*, *7*).

The microtubule cytoskeleton has emerged as an effective target for cancer chemotherapy (*8*), as demonstrated by the clinical effectiveness of Taxol that has been approved by the FDA for the treatment of ovarian, breast, and non-small-cell lung carcinomas. Taxol is an anti-mitotic agent that has the capacity to stabilize microtubules against depolymerization. As with many cancer chemotherapeutic agents, resistance remains a significant problem in the treatment of

† This work was supported in part by USPHS Grants CA 39821 and CA 77263, by the National Foundation for Cancer Research (S.B.H.), AI49749, and by Grant DAMD17-01-0123 from the Department of Defense Breast Cancer Program (G.A.O).

* To whom correspondence should be addressed. Tel.: (718) 430-3742. Fax: (718) 430-8922. E-mail: orr@aecom.yu.edu.

‡ Department of Molecular Pharmacology.

§ Laboratory for Macromolecular Analysis and Proteomics.

+ These authors contributed equally to this work.

[1] Abbreviations used: Da, dalton; ESI-MS, electrospray ionization mass spectrometry; FTICR, Fourier transform ion cyclotron resonance; IEF, isoelectric focusing; LC−MS, liquid chromatography−mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight; MAPs, microtubule-associated proteins; MS/MS, tandem mass spectrometry; RT-PCR, reverse transcriptase−polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

10.1021/bi0350147 CCC: $25.00
Published on Web 09/25/2003

malignancies with Taxol (*9*, *10*). It is not known by what mechanisms human tumors become resistant to Taxol, but it is probable that there is more than one mechanism involved in most tumors.

Microtubules are dynamic, not static, polymers and the precise regulation of this dynamicity is important for many cellular processes (*1*, *11*, *12*). Alterations in microtubule dynamics that occur in the development of resistance to Taxol and other microtubule-stabilizing drugs have become an emerging theme in drug resistance literature (*9*, *10*, *13*, *14*). The dynamics of individual rhodamine-labeled microtubules in Taxol-sensitive and -resistant A549 cell lines, derived from a human lung carcinoma, have been quantified by digital time-lapse microscopy (*15*). The A549-T12 and -T24 cell lines, 9- and 17-fold resistant to Taxol, respectively, were selected by continual exposure of the parental drug-sensitive cell line to increasing concentrations of drug (*23*). The microtubules from both resistant cell lines exhibited increased dynamic instability compared with the parental, drug-sensitive cell line. There are several potential mechanisms by which microtubule dynamics could be modulated in a Taxol-resistant cell line: altered tubulin isotype expression, mutations to tubulin that affect either longitudinal/lateral interactions or binding of regulatory proteins, alterations in post-translational modifications of tubulin that modify regulatory protein binding, and altered expression or post-translational modifications to microtubule regulatory proteins.

Altered expression of tubulin isotypes and mutated tubulins has been detected in cancer cell lines resistant to microtubule-interacting agents. Microtubules consisting of one particular β-tubulin isotype display differential dynamics and exhibit differential sensitivity to the suppressive effects of Taxol on microtubule dynamics (*16*−*19*). There have been numerous reports of altered expression of individual β-tubulin isotypes in cells that have been selected for resistance to antimitotic agents (*20*−*29*). These studies suggest that altered expression of β-tubulin isotypes, especially classes III and IVa, may be correlated with Taxol sensitivity. Mutations in βI-tubulin either within the Taxol-binding pocket or in domains involved in microtubule assembly have been detected in cell lines resistant to microtubule agents (*30*−*33*).

There is a need for rapid, sensitive, and accurate methods for assessing tubulin composition, modifications, and mutations in human cell lines and tissues. We previously reported on the tubulin structural diversity in human cancer cell lines by utilizing negative ion MALDI-TOF MS to analyze the highly acidic C-terminal human tubulin peptides generated by CNBr digestion (*34*). However, this method does not allow the detection of modifications or mutations occurring outside of the extreme C-terminal domain. More recently, we used isoelectric focusing combined with MALDI mass mapping to resolve and characterize tubulin isotypes in human cell lines (*35*). This method allows the analysis of post-translational modifications but limits the detection of mutations to those introducing a change in charge. In this paper, we describe the direct analysis of native tubulin isotypes by LC−MS analysis of microtubules isolated from cell lines sensitive or resistant to Taxol and epothilones.

MATERIAL AND METHODS

Chemicals. Taxol was obtained from the Drug Development Branch of the National Cancer Institute (Bethesda, MD), dissolved in sterile DMSO, and stored at −20 °C. Trypsin was obtained from Promega (Madision, WI). All other chemicals were obtained from Sigma (St. Louis, MO), except where noted.

Cell Culture. A549, a human non-small-cell lung line, and HeLa, a human cervical carcinoma cell line, were maintained as described previously (*23*, *36*).

Isolation of Tubulin from Cell Lines. Taxol-stabilized microtubule pellets were isolated from cytosolic extracts following the method of Vallee (*37*) as described previously (*35*).

LC−MS. Taxol-stabilized microtubules (∼10 μg) were dissolved in 70% formic acid and immediately loaded onto a 1.0- × 150-mm Vydac C_4 column (Vydac, Hesperia, CA) at a flow rate of 50 μL/min. The mobile phases used for protein separations were 5% acetonitrile containing 0.1% TFA (solvent A) and 95% (v/v) acetonitrile containing 0.1% TFA (solvent B). The protein samples were initially desalted with 5% solvent B for 45 min and then separated using either 0.2%/min or 0.05% /min solvent B gradients. For the 0.2% /min gradient, the initial 5% solvent B was increased to 40% over 3 min, followed by 40−60% (100 min) and 60−95% (7 min) gradients. The 0.05%/min gradient was generated by a 5−30% gradient (3 min), followed by 30−45% (17 min), 45−51% (120 min), and 51−75% B (5 min) gradients. The column effluent was delivered directly to a LCQ quadrupole ion trap mass spectrometer (ThermoFinnigan, Riviera Beach, FL). The mass spectrometer was operated in normal MS scan mode to detect ions in the *m*/*z* range of 900−1300.

"In Solution" Trypsin Digestion of HPLC Fractions. Proteins were separated as described above using the 0.05%/min solvent B gradient, except that the column effluent flow (50 μL/min) was split for fraction collection (35 μL/min) and for delivery to the ion trap mass spectrometer (15 μL/min). The appropriate HPLC fractions were pooled and centrifuged by a SpeedVac concentrator to evaporate solvents and to reduce the volume to ca. 10 μL. Twenty microliters of 200 mM ammonium bicarbonate, pH 8.1, was added to each tube, followed by the addition of 6 μL of trypsin (0.05 μg/μL) dissolved in ammonium bicarbonate. Incubations were performed overnight at 37 °C with constant shaking. The digests were desalted by Millipore C18 ZipTip, and the tryptic peptides were eluted with 50% acetonitrile/H_2O solution containing 0.1% TFA (4 μL).

Analysis of Tubulin C-Terminal Peptides. Tubulin was isolated from cells as described previously (*35*). Taxol-stabilized microtubule pellets were solubilized in Laemmli sample buffer, and proteins were separated by SDS−PAGE using running conditions that separate α-tubulin from β-tubulin (*38*). Gels were transferred to nitrocellulose and stained with Ponceau Red, and regions containing either α- or β-tubulin were cut and processed for CNBr digestion and negative-mode MALDI-TOF MS analysis as described previously (*34*).

Protein Identification. MALDI spectra were recorded in the positive or the negative mode on a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA), equipped with a 2.0-m flight tube and a 337-nm nitrogen laser. Saturated α-cyano-4-hydroxycinnamic acid was used as the matrix. Protein identification was accomplished through database searching (Swiss-Prot and

NCBI) using MS-Fit and ProFound programs (*Homo sapiens*/*Mus musculus*, mass tolerance of 1 Da, partially oxidized methionine, average and/or monoisotopic masses, and a maximum of two miscleavages). The mass value for each tubulin isotype was calculated using the Compute MW/p*I* tool from the ExPaSy website.

RESULTS AND DISCUSSION

Alterations in tubulin isotype composition and mutations have been proposed as potential mechanisms of resistance toward microtubule-interacting cytotoxic drugs. However, the structural diversity of mammalian tubulins, involving multiple isotypes with assorted post-translational modifications, makes comparative analysis between sensitive and resistant cell lines difficult. Both RT-PCR product quantitation and sequencing or antibody-based approaches with tubulin isotype-specific antibodies have been exploited to study the tubulin composition in drug-sensitive and -resistant cell lines. However, there is often a poor correlation between mRNA and translated protein levels (*39*, *40*). This is of special relevance when a mutation occurs in a single allele of a specific tubulin gene, since it is important to know whether both wild-type and mutant alleles are expressed and at what levels. No α-tubulin isotype-specific antibodies are readily available, except for those directed against specific post-translational modifications i.e., acetylated, tyrosinated, and non-tyrosinated α-tubulins. Antibodies are available that recognize glutamylated and glycylated α/*β*-tubulins, but these antibodies give no insight into the length of the appended side chain. This is important information since it has been proposed that tubulin polyglutamylation regulates the binding of both structural and motor MAPs as a function of the length of the polyglutamyl side chain (*6*, *7*).

Analysis of Tubulins by Mass Spectrometry. In recent years, mass spectrometry-based approaches have gained in popularity for the analysis of tubulins across distinct phyla, from lower protozoa to mammals (*41*−*50*). These studies have focused largely on the analysis of the C-terminal peptides in order to define the post-translational modifications that occur to this domain of tubulin. In mammalian brain tubulin, polyglutamylation of α-and *β*-tubulin (*47*), phosphorylation of *β*III-tubulin (*41*), reversible tyrosination of α-tubulin (*47*), removal of penultimate glutamate from detyrosinated α-tubulin, and more recently polyglycylation of Δ2-tubulin (*51*) were demonstrated by mass spectrometry. In contrast, studies of mammalian tubulin from non-neuronal sources by mass spectrometry are very few. We recently developed a strategy for the analysis of tubulin isotype composition and their post-translational modifications in A549, a non-small-cell lung cancer cell line, and in MDA-MB-231, a breast carcinoma cell line (*34*). Tubulins present in total cell extracts from these cell lines were analyzed by SDS−PAGE and transferred to nitrocellulose, followed by excision of the tubulin region of the membrane and CNBr digestion. The released peptides were analyzed by negative ion mode MALDI-TOF mass spectrometry to detect selectively the highly acidic C-terminal tubulin peptide ions (*34*, *52*). In these cancer cell lines, the major tubulin isotypes detected were Kα1- and *β*I-tubulin, as well as *β*IVb-tubulin and, at that time, a new human α-tubulin that was named α*. Subsequent work (see below) established that this isotype was the human homologue of mouse α6. In contrast to brain tubulin, Kα1- and *β*I-tubulin were only monoglutamylated, and no other post-translational modifications were detected. However, our inability to detect the C-terminal peptides of *β*II-, *β*III-, and *β*IVa-tubulin isotypes, given that these isotypes were previously identified in A549 cells by Western blotting and/or RT-PCR, revealed a potential limitation to this approach (*23*). With no separation of C-terminal peptides prior to MALDI-TOF mass spectrometry analysis, ions for minor tubulin species could have been suppressed by the presence of other, more abundant C-terminal tubulin peptides. Moreover, the method gave no information regarding potential modifications or mutations outside the C-terminal domains. More recently, we developed a combined isoelectric focusing/mass mapping approach to analyze tubulin isotypes in Taxol-stabilized microtubule preparations (*35*).

Table 1: Calculated Masses of Human Tubulin Isotypes

accession no.[a]	tubulin isotype	mass, Da
	α-Tubulin	
CAA25855	α1/bα1	50 157.7
I77403	α1/Kα1	50 151.6
AAC31959	α1/Kα1	50 151.6
AAD33871	α1/Kα1	50 135.6
Q13748	α3	49 959.5
A25873	α4	49 924.4
Q9BQE3	α6	49 895.3
Q9NY65	α8	50 093.5
	β-Tubulin	
AAD33873	*β*I	49 670.8
P07437	*β*I	49 759.9
AAH01352	*β*II	49 953.1
NP_001060	*β*II	49 907.0
AAH00748	*β*III	50 432.7
NP_006077	*β*III	50 517.8
P04350	*β*IVa	49 630.9
NP_006078	*β*IVa	49 585.8
P05217	*β*IVb	49 831.0
NP_115914	*β*V	49 857.1
NP_110400	*β*VI	50 326.9

[a] NCBI protein database.

Native Tubulin Isotype Masses. Except for two Kα1-tubulin variants, the calculated masses of the human tubulin isotypes found in the databases are all distinct (Table 1). We have used reversed-phase chromatography in line with ESI mass spectrometry analysis to determine the masses of native tubulin isotypes in human cancer cell lines. Prior to analysis, the α/*β*-tubulins were isolated from cell extracts by a Taxol-dependent polymerization process originally developed by Vallee (*37*). We have recently established that there is no selective loss of specific tubulin isotypes during this process (*35*). Therefore, the isolated tubulins are representative of tubulins in cells. The determination of native tubulin masses is important for relating sequences entered in databases to the proteins expressed in human cell lines. This is particularly important in a protein family like tubulin, where nomenclatures and annotations remain confusing and multiple sequence variants of a single tubulin isotype can be found in databases (Table 1). Native mass determinations could eliminate erroneous entries or validate some that could represent true polymorphisms.

Tubulin Isotypes in A549 and HeLa Cell Lines. Proteins present in Taxol-stabilized microtubule pellets from A549 and HeLa cells were analyzed by LC−MS using a microbore C4 reversed-phase column and a 0.2%/min acetonitrile

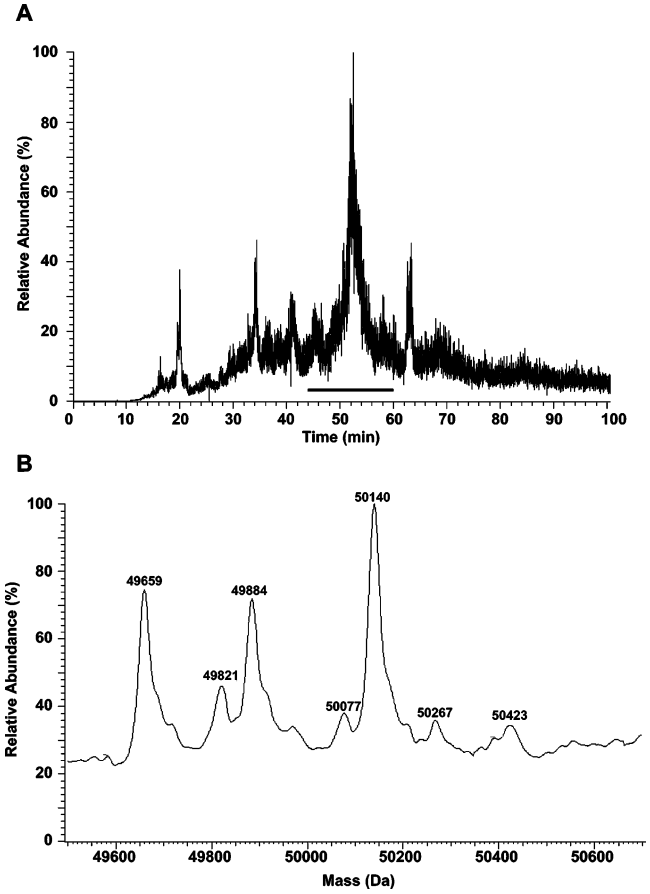


FIGURE 1: Analysis of Taxol-stabilized microtubules from A549 cells by LC−MS. A pellet of A549 Taxol-stabilized microtubules was resuspended in 70% formic acid, and proteins were separated on a microbore C4 reversed-phase column with a 0.2%/min solvent B gradient and detected by ESI-MS. (A) Total ion current profile. (B) Deconvoluted mass obtained by averaging scans in the retention time range indicated by a horizontal black bar in A.

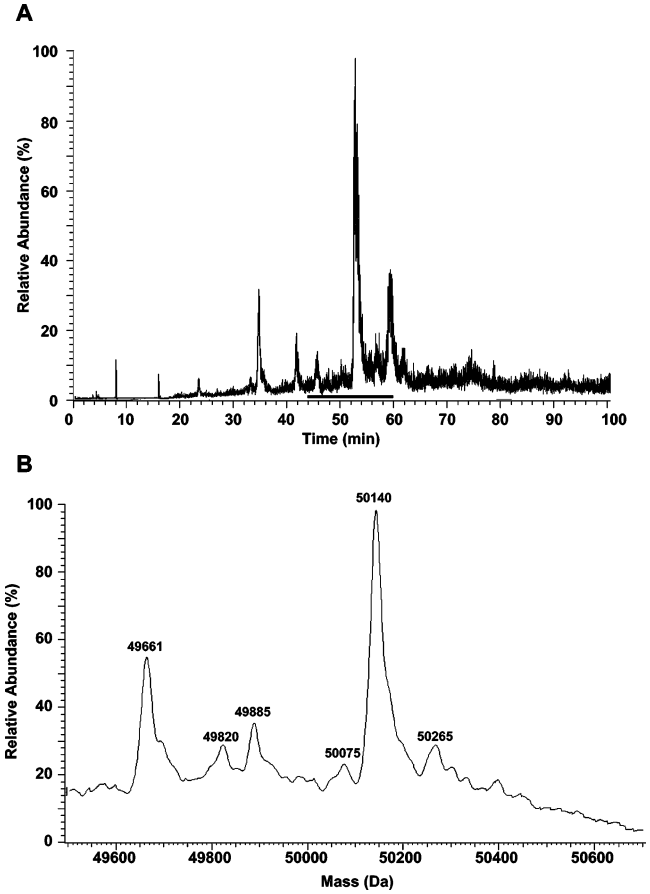


FIGURE 2: Analysis of Taxol-stabilized microtubules from HeLa cells by LC−MS. A pellet of HeLa Taxol-stabilized microtubules was resuspended in 70% formic acid, and proteins were separated on a microbore C4 reversed-phase column with a 0.2%/min solvent B gradient and detected by ESI-MS. (A) Total ion current profile. (B) Deconvoluted mass obtained by averaging scans in the retention time range indicated by a horizontal black bar in A.

gradient. The total ion chromatograms from both cell lines were similar, although the relative intensities of the signals differed (Figures 1A and 2A). All the masses in the range of human tubulins (see Table 1) were found between 44 and 60 min of the gradient, which includes the largest ion peak. This result was expected, as tubulin represented the major protein component in the Taxol-stabilized microtubule pellets. After averaging the scans between 44 and 60 min, we obtained overall profiles of the deconvoluted mass peaks for the different tubulin proteins in both cell lines (Figures 1B and 2B). The observed masses matched closely those of βI-, βIVb-, α6-, tyr α4-, Kα1-, glu Kα1-, and βIII-tubulin, respectively (Table 2).

To confirm the identity of these tubulin isotypes, tryptic mass mapping of the C4-resolved isotypes present in A549 tubulins was performed. For these experiments, tubulin isotypes were separated using a 0.05%/min acetonitrile gradient to increase the separation between tubulin isotypes, and the column effluent was split for fraction collection and for delivery to the ion trap mass spectrometer. With this shallower gradient, the tubulin isotypes eluted between 54 and 96 min. The total ion current was deconvoluted in 1-min segments across the gradient, and fractions that contained the same protein masses were pooled. After removal of solvents, tryptic digestion was performed and the resulting peptides were analyzed by MALDI-TOF MS, searching for

Table 2: Comparison of Experimental and Calculated Masses of Human Tubulin Isoforms

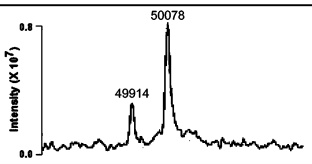
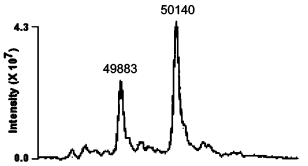
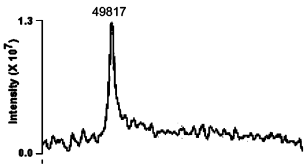
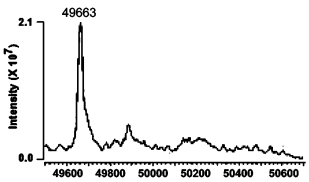
tubulin	mass, Da		Δmass
	calcd[a]	measd[b]	
Kα1	50 151.6	50 142.2 ± 2.2	−9.4
Kα1 glu_1	50 280.7	50 266.0 ± 2.0	−14.0
Kα1 $Ser_{379}Arg$	50 220.7	50 209.5 ± 3.5	−11.2
α6	49 895.3	49 885.6 ± 1.3	−9.7
α4	49 924.4	49 913.3 ± 1.1	−11.1
α4 tyr	50 087.6	50 077.0 ± 1.7	−10.6
βI	49 670.8	49 661.6 ± 0.5	−9.2
βIVb	49 831.0	49 821.2 ± 2.5	−9.8
βIII	50 432.7	50 422.0 ± 3.5	−10.7
βIII glu_1	50 561.8	50 551.0 ± 3.5	−10.8

[a] Calculated from sequences in NCBI protein database (see Table 1). [b] Average of four independent experiments with microtubules isolated from A549 and A549-T12 cell lines ± standard deviation.

tubulin isotype-specific tryptic peptides. The native tubulin masses (Table 3 and Figure 3) and the detected isotype-specific tryptic peptides (Table 3) present in these pooled fractions are as follows:

Pool 1. The 54−56-min segment of the gradient contained a very small peak with an average mass of 49 914 Da and a larger peak with an additional mass of 163 Da. These masses were close to the calculated values for α4- and tyrosinated α4-tubulins, respectively (Table 2). MALDI mass mapping

Table 3: Peptide Mass Mapping of HPLC-Resolved Tubulin Isotypes from A549 Cells

Retention time range (min)	Mass in HPLC fraction (Da)	Isotype-specific tubulin peptides[a]	Measured mass	Calculated mass	Δmass (Da)	Database matched tubulin	Sequence coverage[b] (%)
Pool 1		$_{113}$EIIDPVLDR$_{121}$	1068.5	1068.6	0.1	α4	40
		$_{431}$DYEEVGIDSYEDEDEGEE$_{448}$[c]	2122.1	2122.0	-0.1		
54-56		$_{431}$DYEEVGIDSYEDEDEGEEY$_{448}$[c]	2285.0	2285.2	0.2	α4 Tyr	
Pool 3		$_{423}$EDMAALEKDYEEVGADSADGEDEGEEY$_{449}$[c]	2966.4	2967.0	0.6	α6	42
		$_{423}$EDMAALEKDYEEVGVDSVEGEGEEEGEEY$_{451}$[c]	3237.3	3237.3	0.0	Kα1	52
70-79		$_{423}$EDMAALEKDYEEVGVDSVEGEGEEEGEEY$_{451}$ *(MSO)* [c]	3253.5	3253.3	-0.2		
Pool 4		$_{63}$AVLVDLEPGTMDSVR$_{77}$	1601.9	1601.8	-0.1	βIVb	53
		$_{47}$INVYYNEATGGK$_{58}$	1327.6	1327.6	0.0		
79-89							
Pool 5		$_{283}$ALTVPELTQQVFDAK$_{297}$	1659.4	1659.2	-0.2	βI	54
		$_{363}$MAVTFIGNSTAIQELFK$_{379}$ *(MSO)*	1886.6	1886.2	-0.4		
92-96		$_{20}$FWEVISDEHGIDPTGTYHGDSDLQLDR$_{46}$	3103.0	3103.3	0.3		

[a] Peptides specific to a tubulin isotype that were detected. MSO: oxidized methionine. [b] Sequence coverage was calculated on the basis of all the tryptic peptides detected, i.e., isotype-specific and shared peptides. [c] Detection of these peptides was greatly improved in negative mode.

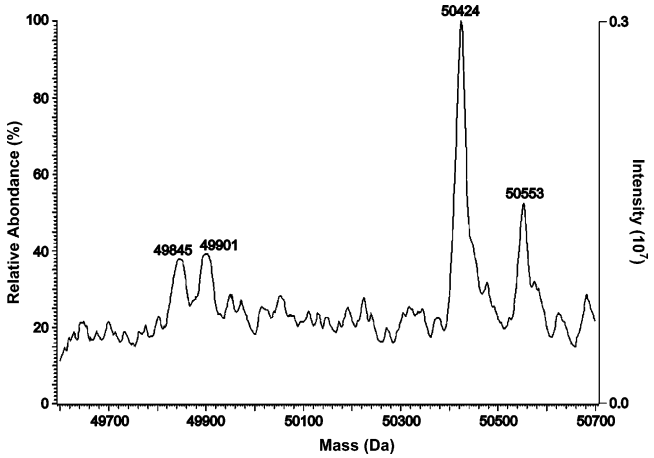


FIGURE 3: Deconvoluted mass spectrum of minor tubulin isoforms. A pellet of A549 Taxol-stabilized microtubules was resuspended in 70% formic acid, and proteins were separated on a microbore C4 reversed-phase column with a 0.05%/min solvent B gradient and detected by ESI-MS. Proteins eluting from 64 to 66 min of retention time (pool 2) were examined for their tubulin isoform content by averaging scans. Two minor mass peaks were tentatively assigned to Δ2 Kα1-tubulin and its monoglycylated isoform. The major peak had a mass matching *β*III-tubulin, and an associated peak corresponding to its monoglutamylated isoform was present.

identified the isotype-specific C-terminal tryptic peptides from both forms, in addition to an internal α4-specific peptide. This is the first evidence of the expression of the α4-tubulin isotype in A549 cells. α4-Tubulin is the only isotype not to have a tyrosine or phenylalanine residue as the last encoded amino acid. However, α4-tubulin is a substrate for tubulin tyrosine ligase, and, once tyrosinated, it can undergo tyrosination/detyrosination cycles like the other α-tubulin isotypes (*5*). We found that the majority of α4-tubulin in A459 and HeLa cells was tyrosinated.

Pool 2. The 64−66-min segment of the gradient contained several minor peaks (Figure 3). First, two peaks that differed in mass by approximately 56 Da were tentatively identified as detyrosinated/deglutamylated (Δ2)-Kα1-tubulin and its monoglycylated counterpart. Δ2-α-Tubulin is unable to undergo the reversible tyrosination cycle (*53*) but has been shown to be glycylated in brain tubulin (*51*). Two other mass peaks that differed by 129 Da were tentatively identified as the *β*III-isotype and its monoglutamylated counterpart. Unfortunately, we could not detect tryptic peptides specific for either Δ2-α- or *β*III-tubulin in this pool, presumably because of their low level of abundance. However, by immunoblot analysis, antibodies reportedly specific for Δ2-α-tubulin cross-reacted weakly with a protein of the correct size in the A549 cell line.[2] We have also detected nonmodified *β*III-tubulin, and a minor more acidic variant, in this cell line by an isoelectric focusing/MALDI mass mapping approach (*35*). This is consistent with the presence of two *β*III-tubulin mass peaks detected by LC−MS.

Pool 3. The 70−79-min segment of the gradient contained two major peaks corresponding to α6- and Kα1-tubulins. The C-terminal tryptic peptide for each isotype was detected. The amino acid sequence obtained previously for the C-terminal peptide of α*-tubulin by MS/MS (*34*) matches the C-terminal sequence of a recently cloned human α6-tubulin. In the present study, a protein having the expected

[2] L. Martello and S. B. Horwitz, unpublished observation.

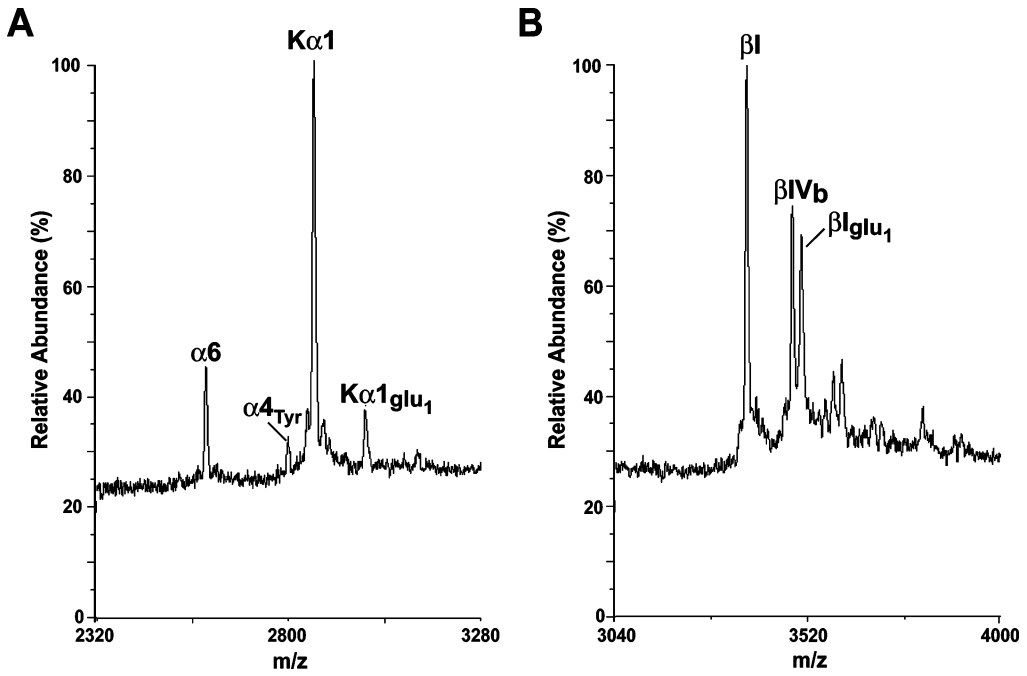


FIGURE 4: Negative ion mode MALDI-TOF analysis of tubulin C-terminal peptides from Taxol-stabilized microtubules isolated from HeLa cells. α-Tubulin and *β*-tubulin were separated by SDS−PAGE and transferred to nitrocellulose, and the α-tubulin band (A) and the *β*-tubulin band (B) were independently CNBr-digested; *m*/*z* peaks were assigned on the basis of the calculated *m*/*z* values for CNBr-generated C-terminal peptides from human tubulin isotypes (*34*).

mass for human α6-tubulin was detected and confirmed as being α6-tubulin by tryptic mass mapping. The observed mass for Kα1-tubulin is close to the three complete sequence variants present in the databases (Table 1). We did not find a tryptic peptide that was specific for any of these Kα1 variants in our mass mapping experiments. However, the Kα1-tubulin nucleotide sequence in A549 deduced by cDNA sequencing (*36*) matches the protein with the accession number AAC31959. Moreover, in our IEF mass mapping study of human tubulins (*35*), we found two tryptic peptides (residues 230−243, *m*/*z* 1487.7 and residues 340−352, *m*/*z* 1527.5) that are unique to this particular Kα1-tubulin variant. Like the α4 isotype, both Kα1- and α6-tubulins in the A549 and HeLa cell lines are present in their tyrosinated forms.

Pool 4. The 79−89-min segment of the gradient contained a single peak with a mass close to that of *β*IVb-tubulin. Two isotype-specific peptides for this tubulin were detected after tryptic digestion.

Pool 5. The 92−96-min segment of the gradient contained a single peak that was shown by mass mapping to be *β*I-tubulin. In our previous studies, we tentatively attributed a low-abundance C-terminus CNBr peptide to monoglutamylated *β*I-tubulin (*34*). Although we did not detect the presence of this modified isotype in A549 by LC−MS, an ion matching monoglutamylated *β*I-tubulin was observed in Taxol-stabilized microtubules from HeLa cells (Figure 6A, below).

A systematic average difference of −10 Da between measured and calculated masses was observed in our data (Table 2). There are 8−12 cysteine residues present in human tubulins, but only two natural disulfide bonds per native α/*β* tubulin heterodimer have been reported, i.e., one disulfide bond per subunit (*54*, *55*). This would account for a 2-Da decrease in mass per α- or *β*-tubulin chain. However, reduction with DTT or tris[2-carboxyethyl]phosphine prior to LC−MS did not alter the observed masses (data not shown). Therefore, the observed mass differences are likely to be due to a systematic calibration error for proteins of this mass range or to disulfide bond interchange during the LC separation stage. To unambiguously address these issues in future studies, it may be helpful to alkylate cysteine residues, before and after reduction, prior to mass analysis.

Unlike mammalian neuronal tubulin, the tubulin isolated from the A549 and HeLa cell lines was not extensively post-translationally modified. However, all of the identified α-tubulin isotypes were mainly tyrosinated. This is consistent with our previous study using MALDI-TOF MS analysis of the C-terminal tubulin peptides from A549 and MDA-MB-231 cell lines (*34*). Acetylation of Lys_{40} is a modification that is also specific to α-tubulins, but none of the α isotypes identified in both cell lines were acetylated, on the basis of their experimentally determined masses and the detection of tryptic peptides with N-terminal Thr_{41} residues in the mass mapping experiments (data not shown). Glutamylation was limited to one residue and was present in a very small fraction of tubulin.

In cells, both detyrosinated and acetylated tubulins are normally associated with stable microtubules (*56*). However, under in vitro assembly conditions, tubulin isolated from HeLa cells was shown to be significantly less dynamic than brain tubulin, which is extensively post-translationally modified (*57*). This suggests that the post-translational status of tubulin has no direct impact on microtubule dynamics and that these modifications are a consequence and not a cause of microtubule stability in vivo. Nevertheless, it is possible that post-translational modifications may affect interactions with regulatory proteins, thus indirectly altering microtubule dynamics (*56*).

Relative Quantitation of Tubulin Isotypes. Although RT-PCR data indicated the presence of *β*II- and *β*IVa-tubulin

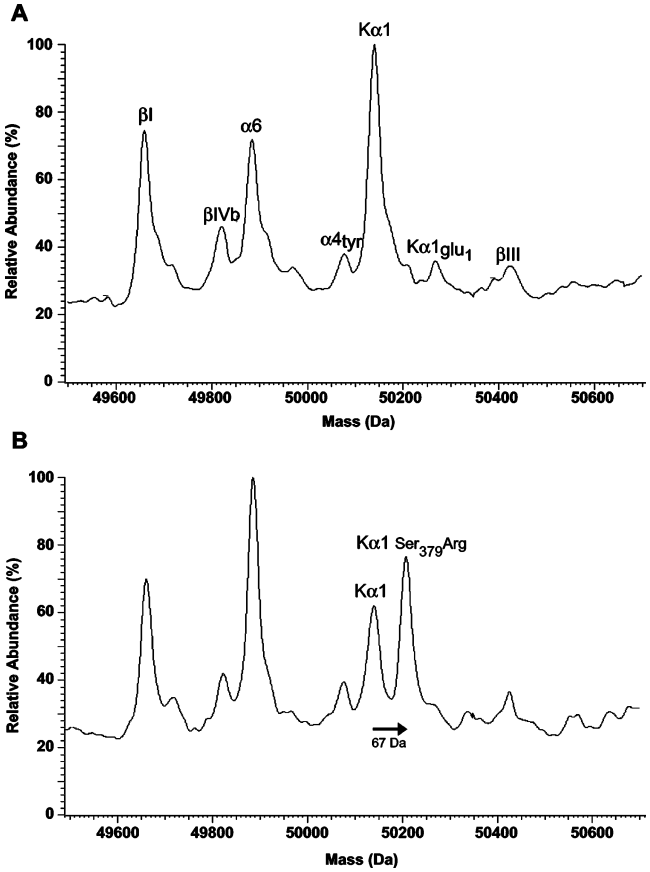


FIGURE 5: Comparison of deconvoluted mass spectra of tubulin isotypes present in A549 and A549-T12. Taxol-stabilized microtubules isolated from A549 (see Figure 1B) and A549-T12 cell lines were analyzed by LC−MS. Mass peaks were assigned on the basis of peptide mapping (Table 3) and calculated values for human tubulin isotypes (Table 2 and Figure 3). Deconvoluted mass profiles were obtained by averaging scans in the retention time range from 44 to 60 min for (A) A549 and (B) A549-T12, where an horizontal arrow indicates a mass shift for a portion of Kα1-tubulin.

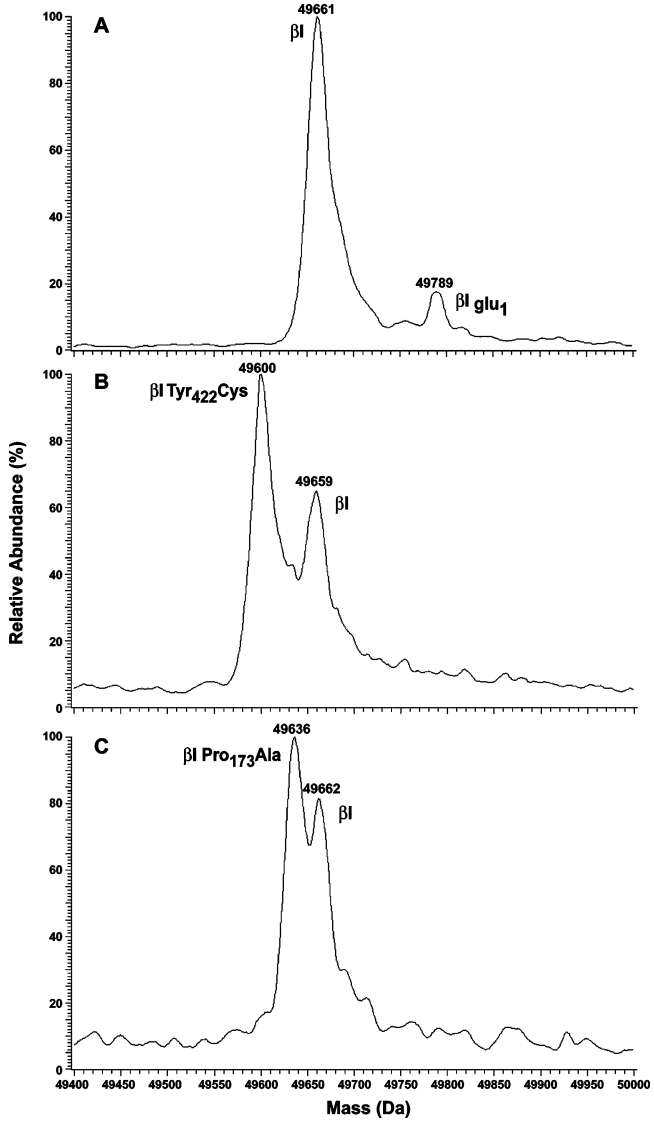


FIGURE 6: Expression of *β*I-tubulin mutations. Taxol microtubules isolated from parental HeLa cells and drug-resistant HeLa cell lines selected with epothilone B (HeLa.EpoB1.8) or with epothilone A (HeLa.EpoA9) were analyzed by LC−MS as described for A549 cell lines. *β*I-Tubulin-deconvoluted mass spectrum from (A) HeLa, (B) HeLa.EpoB1.8, and (C) HeLa.EpoA9 are presented.

mRNA transcripts in the A549 cell line (*23*), the translated proteins were not expressed at detectable levels. Similar results were obtained by MALDI-MS analysis of either the released C-terminal CNBr peptides (*34*) or IEF-resolved tubulin isotypes (*35*). Likewise, *β*II- and *β*IVa-tubulins were below the level of detection in HeLa cells. There is no obvious explanation for the discrepancy between tubulin isotype mRNA and protein expression levels, but such absence of correlation has been observed for other proteins (*39*, *40*). Since tubulin was quantitatively recovered from cells by the Taxol-driven polymerization procedure (*35*), either there is an unknown bias in the RT-PCR results or there is a still unknown silencing mechanism for translation of *β*II- and *β*IVa-tubulin mRNA.

It is known from previous studies that the ratio of total α- to total *β*-tubulin is close to one (*35*). Inspection of the ion intensities in Figures 1B and 2B would suggest, however, that the α-tubulin isotypes are present in considerable excess over the *β*-tubulin isotypes in these cell lines. However, quantitation of relative protein expression levels by comparison of ion intensities is problematic due to differential ionization efficiencies. Nevertheless, if we estimate the relative amounts of individual α- or *β*-tubulin isotypes within each isotype class, we find that Kα1-, α6-, and α4-tubulin or *β*I-, *β*IVb-, and *β*III-tubulin represent approximately 55%, 31%, and 14%, respectively, of total α-tubulin or total *β*-tubulin. These percentages are very similar to those obtained by Coomassie blue staining of the tubulin isotypes resolved by isoelectrofocusing (*35*). It would appear, therefore, that in these human cell lines, Kα1- and *β*I-tubulin, α6- and *β*IVb-tubulin, and α4-and *β*III-tubulin are apparently present in comparable amounts. Because of this trend, we speculate that there may be a sorting mechanism of tubulin isotypes during α/*β*-tubulin heterodimer formation. As stated above, our previous MALDI-TOF MS analysis of tubulin CNBr C-terminal peptides from total cell extracts may have under-represented certain tubulin isotypes due to ion suppression effects. In that study, the C-terminal peptides of *β*II, *β*III, and *β*IVa were not observed, and *β*IVb peptide ion was minor compared to *β*I. We re-evaluated this approach by reducing the complexity of the sample prior to CNBr. In this study, tubulin was purified by the Taxol-based method from HeLa cells, followed by the separation of α-tubulin and *β*-tubulin on SDS−PAGE gels prior to transfer to the nitrocellulose membrane. The region of the blot containing

either α-tubulin or β-tubulin was CNBr-digested, and MALDI-TOF MS analysis was performed. The *m*/*z* peaks' intensities corresponding to βIVb- and monoglutamylated βI-tubulin C-terminal peptides were increased significantly (Figure 4B). Very low levels of the tyrosinated α4-tubulin C-terminal peptide were also detected (Figure 4A), but βIII-tubulin C-terminal peptide was still undetectable (data not shown), indicating a particularly strong suppression of this peptide. Similar results were obtained with A549 tubulin (data not shown). Our results are also consistent with the Western blot analysis of purified HeLa tubulin by Newton et al. (*57*), where βI- and βIV-tubulin isotypes represented 80% and 20% of β-tubulin, respectively, and no βIII-tubulin was detected.

Mutations in Kα1- and βI-Tubulins. Mutations to the primary drug target are a recurring theme in drug resistance. However, DNA sequence analysis of the multiple tubulin genes in human cell lines and tissues is not straightforward since they are so highly conserved. A recent study identified β-tubulin mutations in serum DNA isolated from 33% of patients with non-small-cell lung cancer (*58*). This finding was considered extremely significant, since it was thought to validate in vitro data from numerous laboratories documenting the acquisition of mutations in Taxol- and epothilone-resistant cell lines that correlated with increasing levels of resistance. Several groups sought to confirm this initial study (*59*−*61*); however, the presence of mutations has not been corroborated in these prospective studies, although silent polymorphisms have been reported. The study by Monzo et al. (*58*), which utilized genomic DNA that was extracted from circulating tumor DNA, isolated from patient serum samples, and the existence of tubulin pseudogenes, makes it difficult to analyze the precise nucleotide sequence of β-tubulin using genomic DNA.

Since the drug-binding site for the microtubule-stabilizing drugs resides in β-tubulin, the search for tubulin mutations in Taxol/epothilones-resistant cell lines has been largely restricted to the major βI-tubulin transcript (*30*−*33*). However, recent studies have shown that mutations to α-tubulin may also occur in some drug-resistant cell lines (*36*). The complete sequencing of all tubulin isotypes in drug-selected cell lines, although feasible, would be very time-consuming. Moreover, sequencing of the α/β-tubulin genes from resistant cell lines has shown that mutations introduced often occur to a single tubulin allele (*30*). Therefore, the question as to whether both the wild-type and mutant transcripts are translated, and to what levels, is important, since the ratio of wild-type to mutant protein could affect resistance levels.

A549-T12, a Taxol-resistant cell line, was reported to have a heterozygous mutation at amino acid 379 of Kα1-tubulin, resulting in a serine-to-arginine substitution (*36*). The mass spectrum of tubulins isolated from this resistant cell line clearly shows a new protein peak approximately 67 Da greater than that of the parental Kα1 isotype (Figure 5). The difference in mass is very close to the expected 69.1 Da due to the Ser-to-Arg substitution at position 379. We also analyzed Taxol-stabilized microtubules from two HeLa cell lines that are resistant to epothilones. Both cell lines were shown to harbor different heterozygous mutations in βI (*33*). The βI-tubulins from these cell lines, HeLa.EpoB1.8 with a Tyr422Cys mutation and HeLa.EpoA9 with a Pro173Ala mutation, were predicted to have masses 60 and 26 Da, respectively, lower than the wild-type isotype. These mass losses were clearly detected in the deconvoluted mass spectra of regions of the gradient at which βI-tubulin elutes (Figure 6). In the parental HeLa cells, we observed only the wild-type βI-tubulin and a small amount of monoglutamylated βI-tubulin (Figure 6A). In the resistant cell lines, the expression of the mutant βI-tubulins was clearly evident (Figure 6B,C), in both cases at levels that appeared higher than the wild-type βI-tubulin.

What is the smallest mass difference between specific tubulin isotypes that can be detected using a mass spectrometer? In this study, we were able to discriminate mutant and wild-type βI isotypes that differed by 26 Da, even though they were incompletely (30%) resolved. Theoretically, the peak width at 50% of maximum peak height is about 15 Da for an ∼50 kDa protein, as estimated by calculating the width of the isotopic distribution using the IsoPro program (http://members.aol.com/msmssoft/). Therefore, two 50-kDa proteins of equal abundance must differ in mass by more than 15 Da to be resolved. It is likely that the use of quadrupole TOF or FTICR mass spectrometers would permit the detection of tubulin mutants displaying even smaller mass differences. Moreover, the presence of a broad or asymmetrical peak, in which the peak full width at 50% of maximum peak height exceeds 15 Da by FTICR MS, would indicate that incompletely resolved tubulin isotypes/mutants were present. In that case, the protein can be collected off-line for enzymatic digestion and amino acid sequence analysis by LC−MS/MS. In studies performed so far, we have achieved almost complete sequence coverage of the major tubulin isotypes by MS/MS analysis of pepsin-derived overlapping peptide fragments.[3]

The ability to perform simultaneous analysis of the expression of tubulin isotypes in human cell lines will have a major impact on microtubule biology. As noted in Table 1, multiple tubulin sequences are present in the databases. By using the present LC−MS approach and/or the IEF-MS methodology described previously, human tubulin sequences that are expressed should be identifiable. This will improve the annotation of tubulin sequences in databases and provide a reference for the detection of potential mutations and polymorphisms in humans. For example, in A549 and HeLa cell lines, we have unambiguously identified expression of Kα1, βI, and βIII with accession numbers AAC31959, AAD33873, and AAH00748, respectively. Likewise, although alterations in tubulin isotype mRNA levels have been noted in cell lines resistant to anti-microtubule agents, our proteomic strategies will afford the opportunity to directly determine the tubulin isotype composition of these cell lines to ascertain whether the mRNA alterations are actually reflected at the protein level. Finally, comparative analysis of tubulin isotype expression and associated post-translational modifications in diseased and normal tissue would provide greater insight into pathologies where the microtubule cytoskeleton appears to be involved (*62*−*66*).

ACKNOWLEDGMENT

We thank Dr. Alan G. Marshall (National High Magnetic laboratory, Florida State University, Tallahassee, FL) for helpful discussions.

[3] F. Wang, unpublished observation.

REFERENCES

1. Desai, A., and Mitchison, T. J. (1997) *Annu. Rev. Cell Dev. Biol. 13*, 83−117.
2. Oakley, B. R. (2000) *Trends Cell Biol. 10*, 537−542.
3. Sharp, D. J., Rogers, G. C., and Scholey, J. M. (2000) *Nature 407*, 41−47.
4. Ludueña, R. F. (1998) *Int. Rev. Cytol. 178*, 207−275.
5. MacRae, T. H. (1997) *Eur. J. Biochem. 244*, 265−278.
6. Boucher, D., Larcher, J. C., Gros, F., and Denoulet, P. (1994) *Biochemistry 33*, 12471−12477.
7. Bonnet, C., Boucher, D., Lazereg, S., Pedrotti, B., Islam, K., Denoulet, P., and Larcher, J. C. (2001) *J. Biol. Chem. 276*, 12839−12848.
8. He, L., Orr, G. A., and Horwitz, S. B. (2001) *Drug Discovery Today 6*, 1153−1164.
9. Dumontet, C., and Sikic, B. I. (1999) *J. Clin. Oncol. 17*, 1061−1070.
10. Drukman, S., and Kavallaris, M. (2002) *Int. J. Oncol. 21*, 621−628.
11. Wilson, L., and Jordan, M. A. (1995) *Chem. Biol. 2*, 569−573.
12. Jordan, M. A., and Wilson, L. (1998) *Curr. Opin. Cell Biol. 10*, 123−130.
13. Cabral, F. R., Brady, R. C., and Schibler, M. J. (1986) *Ann. N.Y. Acad. Sci. 466*, 745−756.
14. Cabral, F., and Barlow, S. B. (1989) *FASEB J. 3*, 1593−1599.
15. Gonçalves, A., Braguer, D., Kamath, K., Martello, L., Briand, C., Horwitz, S., Wilson, L., and Jordan, M. A. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 11737−11742.
16. Lu, Q., and Ludueña, R. F. (1993) *Cell Struct. Funct. 18*, 173−182.
17. Lu, Q., and Ludueña, R. F. (1994) *J. Biol. Chem. 269*, 2041−2047.
18. Panda, D., Miller, H. P., Banerjee, A., Ludueña, R. F., and Wilson, L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11358−11362.
19. Derry, W. B., Wilson, L., Khan, I. A., Ludueña, R. F., and Jordan, M. A. (1997) *Biochemistry 36*, 3554−3562.
20. Carles, G., Braguer, D., Dumontet, C., Bourgarel, V., Gonçalves, A., Sarrazin, M., Rognoni, J. B., and Briand, C. (1999) *Br. J. Cancer 80*, 1162−1168.
21. Dumontet, C., Duran, G. E., Steger, K. A., Beketic-Oreskovic, L., and Sikic, B. I. (1996) *Cancer Res. 56*, 1091−1097.
22. Haber, M., Burkhart, C. A., Regl, D. L., Madafiglio, J., Norris, M. D., and Horwitz, S. B. (1995) *J. Biol. Chem. 270*, 31269−31275.
23. Kavallaris, M., Kuo, D. Y., Burkhart, C. A., Regl, D. L., Norris, M. D., Haber, M., and Horwitz, S. B. (1997) *J. Clin. Invest. 100*, 1282−1293.
24. Ranganathan, S., Dexter, D. W., Benetatos, C. A., Chapman, A. E., Tew, K. D., and Hudes, G. R. (1996) *Cancer Res. 56*, 2584−2589.
25. Ranganathan, S., Benetatos, C. A., Colarusso, P. J., Dexter, D. W., and Hudes, G. R. (1998) *Br. J. Cancer 77*, 562−566.
26. Kyu-Ho Han, E., Gehrke, L., Tahir, S. K., Credo, R. B., Cherian, S. P., Sham, H., Rosenberg, S. H., and Ng, S. (2000) *Eur. J. Cancer 36*, 1565−1571.
27. Nicoletti, M. I., Valoti, G., Giannakakou, P., Zhan, Z., Kim, J. H., Lucchini, V., Landoni, F., Mayo, J. G., Giavazzi, R., and Fojo, T. (2001) *Clin. Cancer Res. 7*, 2912−2922.
28. Burkhart, C. A., Kavallaris, M., and Horwitz, S. B. (2001) *Biochim. Biophys. Acta 1471*, O1−O9.
29. Banerjee, A. (2002) *Biochem. Biophys. Res. Commun. 293*, 598−601.
30. Giannakakou, P., Sackett, D. L., Kang, Y. K., Zhan, Z., Buters, J. T., Fojo, T., and Poruchynsky, M. S. (1997) *J. Biol. Chem. 272*, 17118−17125.
31. Giannakakou, P., Gussio, R., Nogales, E., Downing, K. H., Zaharevitz, D., Bollbuck, B., Poy, G., Sackett, D., Nicolaou, K. C., and Fojo, T. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 2904−2909.
32. Poruchynsky, M. S., Giannakakou, P., Ward, Y., Bulinski, J. C., Telford, W. G., Robey, R. W., and Fojo, T. (2001) *Biochem. Pharmacol. 62*, 1469−1480.
33. He, L., Yang, C. P., and Horwitz, S. B. (2001) *Mol. Cancer Ther. 1*, 3−10.
34. Rao, S., Aberg, F., Nieves, E., Horwitz, S. B., and Orr, G. A. (2001) *Biochemistry 40*, 2096−2103.
35. Verdier-Pinard, P., Wang, F., Martello, L., Burd, B., Orr, G. A., and Horwitz, S. B. (2003) *Biochemistry 42*, 5349−5357.
36. Martello, L. A., Verdier-Pinard, P., Shen, H. J., He, L., Torres, K., Orr, G. A., and Horwitz, S. B. (2003) *Cancer Res. 63*, 1207−1213.
37. Vallee, R. B. (1986) *Methods Enzymol. 134*, 104−115.
38. Rao, S., Krauss, N. E., Heerding, J. M., Swindell, C. S., Ringel, I., Orr, G. A., and Horwitz, S. B. (1994) *J. Biol. Chem. 269*, 3132−3134.
39. Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999) *Mol. Cell Biol. 19*, 1720−1730.
40. Chen, G., Gharib, T. G., Huang, C. C., Taylor, J. M., Misek, D. E., Kardia, S. L., Giordano, T. J., Iannettoni, M. D., Orringer, M. B., Hanash, S. M., and Beer, D. G. (2002) *Mol. Cell Proteomics 1*, 304−313.
41. Alexander, J. E., Hunt, D. F., Lee, M. K., Shabanowitz, J., Michel, H., Berlin, S. C., MacDonald, T. L., Sundberg, R. J., Rebhun, L. I., and Frankfurter, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4685−4689.
42. Bre, M. H., Redeker, V., Vinh, J., Rossier, J., and Levilliers, N. (1998) *Mol. Biol. Cell 9*, 2655−2665.
43. Mary, J., Redeker, V., Le Caer, J. P., Rossier, J., and Schmitter, J. M. (1996) *J. Biol. Chem 271*, 9928−9933.
44. Mary, J., Redeker, V., Le Caer, J. P., Rossier, J., and Schmitter, J. M. (1997) *J. Protein Chem. 16*, 403−407.
45. Redeker, V., Levilliers, N., Schmitter, J. M., Le Caer, J. P., Rossier, J., Adoutte, A., and Bre, M. H. (1994) *Science 266*, 1688−1691.
46. Redeker, V., Rusconi, F., Mary, J., Prome, D., and Rossier, J. (1996) *J. Neurochem. 67*, 2104−2114.
47. Redeker, V., Rossier, J., and Frankfurter, A. (1998) *Biochemistry 37*, 14838−14844.
48. Rudiger, A., Rudiger, M., Weber, K., and Schomburg, D. (1995) *Anal. Biochem. 224*, 532−537.
49. Rudiger, M., Plessmann, U., Rudiger, A. H., and Weber, K. (1995) *FEBS Lett. 364*, 147−151.
50. Vinh, J., Langridge, J. I., Bre, M. H., Levilliers, N., Redeker, V., Loyaux, D., and Rossier, J. (1999) *Biochemistry 38*, 3133−3139.
51. Banerjee, A. (2002) *J. Biol. Chem. 277*, 46140−46144.
52. Jai-nhuknan, J., and Cassady, C. J. (1998) *Anal. Chem. 70*, 5122−5128.
53. Lafanechere, L., and Job, D. (2000) *Neurochem. Res. 25*, 11−18.
54. Chaudhuri, A. R., Khan, I. A., and Ludueña, R. F. (2001) *Biochemistry 40*, 8834−8841.
55. Ikeda, Y., and Steiner, M. (1978) *Biochemistry 17*, 3454−3459.
56. Palazzo, A., Ackerman, B., and Gundersen, G. G. (2003) *Nature 421*, 230.
57. Newton, C. N., DeLuca, J. G., Himes, R. H., Miller, H. P., Jordan, M. A., and Wilson, L. (2002) *J. Biol. Chem. 277*, 42456−42462.
58. Monzo, M., Rosell, R., Sanchez, J. J., Lee, J. S., O'Brate, A., Gonzalez-Larriba, J. L., Alberola, V., Lorenzo, J. C., Nunez, L., Ro, J. Y., and Martin, C. (1999) *J. Clin. Oncol. 17*, 1786−1793.
59. Kelley, M. J., Li, S., and Harpole, D. H. (2001) *J. Natl. Cancer Inst. 93*, 1886−1888.
60. Sale, S., Oefner, P. J., and Sikic, B. I. (2002) *J. Natl. Cancer Inst. 94*, 776−777; discussion 777.
61. Tsurutani, J., Komiya, T., Uejima, H., Tada, H., Syunichi, N., Oka, M., Kohno, S., Fukuoka, M., and Nakagawa, K. (2002) *Lung Cancer 35*, 11−16.
62. Sato, K., and Abe, K. (2001) *Brain Res. 904*, 157−160.
63. Schuller, E., Gulesserian, T., Seidl, R., Cairns, N., and Lube, G. (2001) *Life Sci. 69*, 263−270.
64. Vijayan, S., El-Akkad, E., Grundke-Iqbal, I., and Iqbal, K. (2001) *FEBS Lett. 509*, 375−381.
65. Garcia, M. L., and Cleveland, D. W. (2001) *Curr. Opin. Cell Biol. 13*, 41−48.
66. Parvari, R., Hershkovitz, E., Grossman, N., Gorodischer, R., Loeys, B., Zecic, A., Mortier, G., Gregory, S., Sharony, R., Kambouris, M., Sakati, N., Meyer, B. F., Al Aqeel, A. I., Al Humaidan, A. K., Al Zanhrani, F., Al Swaid, A., Al Othman, J., Diaz, G. A., Weiner, R., Khan, K. T., Gordon, R., and Gelb, B. D. (2002) *Nat. Genet. 32*, 448−452.

BI0350147